Pak. J. Bot., 48(6): 2433-2438, 2016.

CONVENTIONAL BREEDING ENHANCES GENETIC DIVERSITY IN *PISUM SATIVUM* (L.) DETECTED THROUGH SDS-PAGE

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Abstract

In the present study, twenty homozygous segregante of edible pea were developed through conventional breeding (2004- 2015). The seed storage proteins were extracted from the seeds of each line and run through Slab-vertical gel Electrophoresis. After staining and de-staining the Electrophoregram was divided into two zones (R-I and R-II). The R-1 was consists of five bands (B-1, 2, 3, 4 & 5) ranging from 180KDa to 34KDa, which calculated < 1% genetic diversity (GD). Out of which B-1, 2 and 3 were polymorphic, while B-4 and B-5 were monomorphic. Due to strong genetic association and low level of GD, the R-I divided the total germplasm into two clusters. Similarly, R-II was comprised of nine bands ranging from 33KDa -10KDa. All the loci were polymorphic and showed >39% genetic diversity. The R-II divided the total lines into four clusters. In cluster plating, 43.74% lines were grouped in C-03, 30.43% in C-01, 13.39% in C-04 and 8.69% in C-02. Variation in bands profile and multiple clustering authenticates that conventional plant breeding enhances the level of genetic variation in seed storage protein profile in pea germplasm detected through SDS-PAGE.

Key words: Peas *homo*-segregante, Biochemical markers, Diversity, Cluster analysis

Introduction

In the 21st century agricultural scientists are facing a number of challenges. Population of the world will expectedly be over 8 billion by 2025 and 9 billion by the end of 2050 (Dyson, 1999). This increase in population are limiting crop yields, expanding or emerging pathogens in insect pests, increase in scarcity of water, limiting arable land, and decreasing labor in agricultural (Khush, 1999). In order to produce enough food for the population of world a substantial correlative effort by agricultural scientists should be needed.

Plant breeding will surely play a major role to meet the feeding requirements of the global population by introducing new varieties with increased yields or particular traits that provide yield stability (Dobermann, 2013). In fact Plant breading is a way to enhance genetic diversity within population (Forster *et al*., 2014) and it is widely recognized that the presence and estimation of genetic diversity is necessary for successful crop improvement in terms of broadening and maintaining the diversity of the genetic base, improving opportunities for selection of improved genetics to meet agricultural production challenges, cultivar identification, abolishes duplicates in the gene stock, establishes the core collection of germplasm and selection of population for genome mapping experiments (Ghafoor & Ahmad 2005; Nisar & Ghafoor, 2010; Wadood *et al*., 2016). Furthermore the production of more and stable yield is directly proportional to genetically heterogeneous populations (Ali *et al*., 2007).

Over the last few decades, estimation of genetic diversity in several crop species has dependent on morphological traits (Nisar *et al*., 2016), but now it is widely documented that genetic diversity evaluation through biochemical methods have several advantages over the classical morphology (Ndiaye *et al.,* 20011). SDS-PAGE is one among most widely used biochemical techniques in describing the genetic structure of several crop plants because of its validity, simplicity and free of environmental fluxes (Ghafoor *et al*., 2005). Researchers approved that biochemical analysis, precisely electrophoretic analysis of seed proteins revealed by SDS-PAGE have provided valid evidence for detecting intraspecific variation and assessing interspecific relationships (Hameed *et al.,* 2009; Irfan *et al.,* 2010).

In the light of our hypothesis that monohybrid cross changed the genetic pattern of edible pea and enhance genetic polymorphism in seed storage protein profile. In this connection 20 homozygous novel segregantes were developed to explore that conventional plant breeding enhances genetic diversity in *Pisum sativum*.

Materials and Methods

Plant material: The present study was carried out on twenty three pea lines in order to study genetic diversity at seed storage protein profile using SDS PAGE. Out of twenty three lines, twenty *homo*-segregantes of *Pisum sativum* developed through single seed descent method from the cross of Fallon[er] and 11760-3[ER] (Nisar & Ghafoor, 2010). After crossing the progeny were consecutively sown in the research area of Malakand University (2005-2015) for the selection of homozygous segregantes. In the experiment Climex was used as check cultivar (PL-22) for comparative performance.

Seed storage protein profiling: To achieve the clime that plant breeding enhances genetic heterogeneity in seed storage profile in edible pea (*Pisum sativum* L.). The seed of each line was crashed into a fine powder. About 400µl of Protein Extraction Buffer (PEB) was added to 0.01 g of powder and mixed well in 1.5ml Eppendorf tube with the help of Vortexer. The extraction buffer (pH 6.8) contained: 0.5 M Tris-HCl, 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bromophenol Blue (BPB) was added to the protein extraction buffer as tracking dye to observe the movement of protein in the gel. The crude homogenate were then centrifuged at 13000 rpm for 10

minutes at 10°C. The extracted protein samples were collected as supernatant and pellets were discarded and then the samples were then kept in refrigerator**.**

Electrophoresis: Vertical gel electrophoresis assembly was used for protein separation. A total of 12µl of PEB was loaded into the gel. The gel consists of two parts: stacking gel (upper gel, 4.5% acrylamide) and separating gel (main gel, 12.5% acrylamide). Electrophoresis was carried out at 100 volt (120 mA) for 3 hours to separate seed storage protein according to their molecular weight. The molecular weights of the dissociated polypeptides were determined by using molecular weight protein standards "Thermo scientific Page Ruler Prestained protein ladder".

The gels was stained (Methanol 440 ml, Acetic acid 60 ml, distilled water 500 ml and CBB2.25g) for 15min and de-staining solution (Methanol 200 ml, acetic acid 50 ml and distilled water 750 ml) over night to observed the clear protein banding profile for data analysis.

Data analysis: After de-staining, the gel was divided into two zones (Zone-I and Zone-II) based on the number of bands/loci. Zone-1 was started from band-1 – band-5 and Zone-II from band-6 – band-14. The binary data matrix was developed. In the binary data matrix the presence of band/loci was scored as (1) and the absence of band/loci tagged as (0). The binary data matrix was subjected to statistical software. The cluster plating was performed through PC-ORD ver.5 software. While, the genetic distances among the lines were computed through STATICTICA vir6.

Results

Banding profiling: In the present study it was proposed that plant breeding enhances the level of genetic diversity in seed protein profile in pea. To achieve this objective, the electropherogram was divided into two zones (R-I and R-II). R-1 was comprised of five bands (B-1 to B-5) ranging from 180KDa to 34KD and R-II started from B-6 – B-14 ranging from 33KDa to 10KDa (Fig. 1). It was observed that in three lines (PL-9, PL-20 and PL-21) the B-1 was absent, which showed 14.28% genetic diversity (GD). Similarly, B-2 was absent in PL-9, while B-3 showed diversity in PL-9 and PL-12 and calculated 4.76 and 9.52% GD respectively. On the other hand B-4 and B-5 were present in all the tested germplasm and selected as monomorphic bands (Fig. 2).

The R-II was comprised of 9 bands ranging from 33 - 10kDa, out of which, B-6, B-12, B-13 and B-14 calculated 43.5, 91.3, 69.6 and 73.9% genetic polymorphism/ diversity. The entire band loci were polymorphic and the lowest polymorphism was recorded in B-08 and B-10 respectively (Fig. 3).

Genetic index: The binary data matrix developed from seed storage protein profile was used to calculate the genetic index among 23 lines. In zone-wise analysis, in the R-I a total of 0.096 (9.6%) genetic diversity was observed. Furthermore, PL-9 and PL-12 showed 0.48 (48%) and 0.24 (24%) genetic disagreement respectively, while the GD value for the remaining lines was lower (Table 1). The R-II calculated an average of 0.39 (39%) GDs for 23 pea lines based on seed storage protein profiles. In the R-II region, PL-8, PL-09, PL-12, PL-16 and PL-17 showed ≥ 0.40 (40%) genetic disagreement (Table 1). The combined genetic distances of R-I and R-II for 23 peas lines were also calculate to see the level of genetic diversity bring through monohybrid cross. A total of 0.489 (48%) genetic disagrement among 23 peas lines were recorded. The parantal lines and cultivar (climex) was genetically diferent from each other at ≥ 0.45 (45%) genetic disagrement level. It was found that the pea lines PL-8, PL-9, PL-12, PL-16, PL-17, PL-19 also showed ≥ 0.45 (45%) genetic diversity and hence selected as genetically diverse lines in the present study (Table 2).

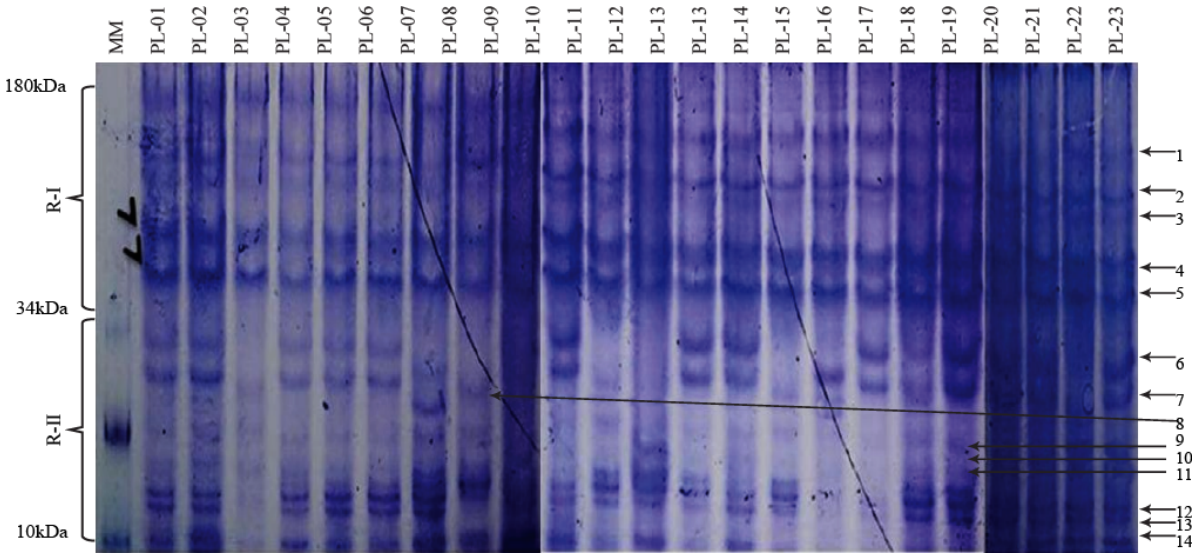

Fig. 1. The seed storage protein profile of 23 pea lines. The arrow indicates the location of bands in the electropherogram. MM-molecular weight marker; R-I zone-1 comprised of B-1 to B-5 ranging from 180 – 34kDa; R-II starting from B-6 – B-14 ranged 33kDa to 10kDa; PL-21 (Pollen/Male); PL-22 Cultivar; PL-23 (Female).

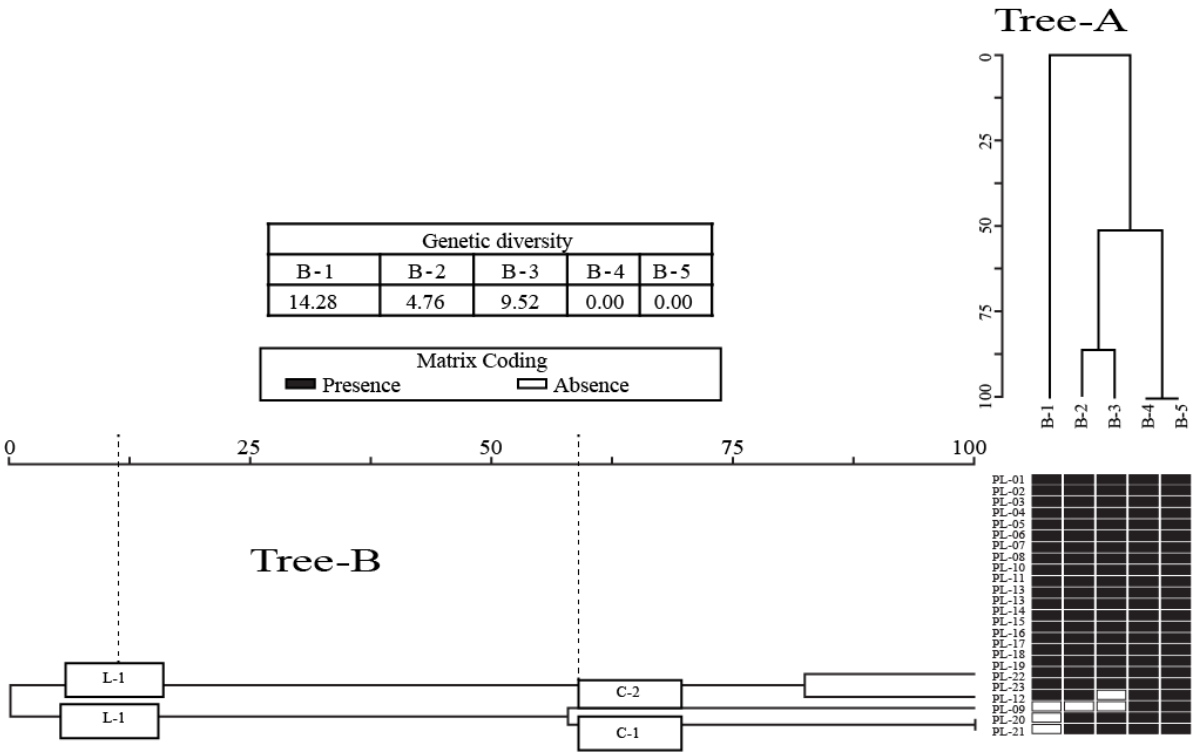

Fig. 2. Two-way Cluster analysis based on seed storage protein profile in 23 pea lines for R-I. Tree-A represents the banding profile from B-01 to B-5; Tree-B showing genetic relationship of 23 peas lines based on 5 bands (B-01 – B-5); the table showing genetic diversity in 5 bands based on bands presence/absence.

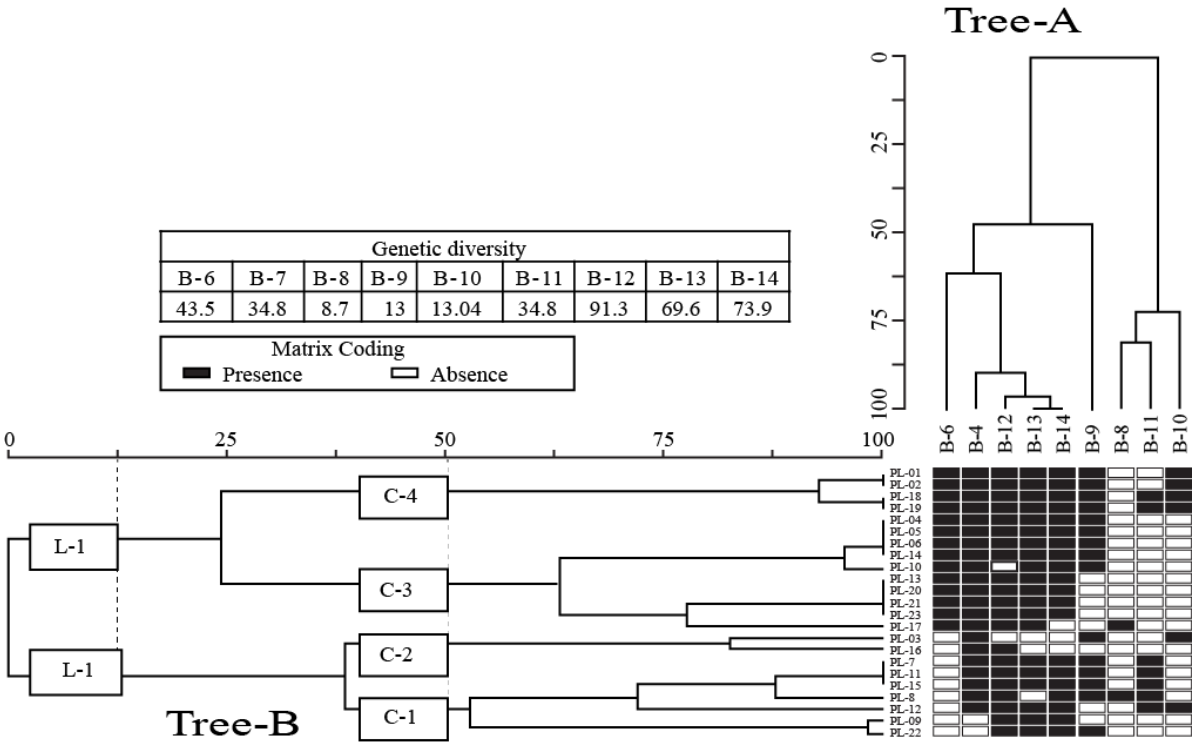

Fig. 3. Two-way Cluster Analysis based on seed storage protein profile in 23 pea lines for R-II. Tree-A represents the banding profile from B-06 to B-14; Tree-B showing genetic relationship of 23 peas lines based on 9 bands (B-06 – B-14); the table showing genetic diversity in 9 bands based on bands presence/absence.

Table 1. The Joining results of 23 pea lines; the amalgamation (joining) rules was unweighted pair-group average (UPGMA) calculated the binary data matric distances in percent disagreement.

PL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	*R-I*
1		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.5	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.05***
2	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.5	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.06***
3	0.20	0.20		0.00	0.00	0.00	0.00	0.00	0.60	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.06***
4	0.20	0.20	0.40		0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.06***
5	0.20	0.20	0.40	0.00		0.00	0.00	0.00	0.40	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.07***
6	0.20	0.20	0.40	0.00	0.00		0.00	0.00	0.40	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.07***
7	0.40	0.40	0.20	0.20	0.20	0.20		0.00	0.40	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.08***
8	0.60	0.60	0.40	0.40	0.40	0.40	0.20		0.50	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.08***
9	0.70	0.70	0.50	0.60	0.60	0.50	0.30	0.50		0.60	0.50	0.40	0.60	0.60	0.50	0.60	0.60	0.60	0.60	0.30	0.40	0.50	0.50	***0.48****
10	0.20	0.20	0.40	0.00	0.00	0.00	0.20	0.40	0.50		0.00	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.05***
11	0.40	0.40	0.20	0.20	0.20	0.20	0.00	0.20	0.30	0.20		0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.05***
12	0.40	0.40	0.20	0.60	0.60	0.60	0.40	0.60	0.30	0.60	0.40		0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.40	0.40	0.20	0.20	***0.24***
13	0.40	0.40	0.60	0.20	0.20	0.20	0.40	0.60	0.30	0.20	0.40	0.40		0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.04***
14	0.20	0.20	0.40	0.00	0.00	0.00	0.20	0.40	0.50	0.00	0.20	0.60	0.20		0.00	0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.04***
15	0.40	0.40	0.20	0.20	0.20	0.20	0.00	0.20	0.30	0.20	0.00	0.40	0.40	0.20		0.00	0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.05***
16	0.60	0.60	0.40	0.40	0.40	0.40	0.20	0.40	0.20	0.40	0.20	0.20	0.20	0.40	0.20		0.00	0.00	0.00	0.20	0.20	0.00	0.00	***0.06***
17	0.60	0.60	0.80	0.40	0.40	0.40	0.60	0.40	0.40	0.40	0.60	0.60	0.20	0.40	0.60	0.40		0.00	0.00	0.20	0.20	0.00	0.00	***0.07***
18	0.00	0.00	0.20	0.20	0.20	0.20	0.40	0.60	0.60	0.20	0.40	0.40	0.40	0.20	0.40	0.60	0.60		0.00	0.20	0.20	0.00	0.00	***0.08***
19	0.00	0.00	0.20	0.20	0.20	0.20	0.40	0.60	0.70	0.20	0.40	0.40	0.40	0.20	0.40	0.60	0.60	0.00		0.20	0.20	0.00	0.00	***0.10***
20	0.40	0.40	0.60	0.20	0.20	0.20	0.40	0.60	0.30	0.20	0.40	0.40	0.00	0.20	0.40	0.20	0.20	0.40	0.40		0.00	0.20	0.20	***0.13***
21	0.40	0.40	0.60	0.20	0.20	0.20	0.40	0.60	0.30	0.20	0.40	0.40	0.00	0.20	0.40	0.20	0.20	0.40	0.40	0.00		0.20	0.20	***0.20***
22	0.60	0.60	0.40	0.40	0.40	0.40	0.20	0.40	0.20	0.40	0.20	0.60	0.60	0.40	0.20	0.40	0.80	0.60	0.60	0.60	0.60		0.00	***0.00***
23	0.20	0.20	0.40	0.40	0.40	0.40	0.60	0.40	1.00	0.40	0.60	0.60	0.60	0.40	0.60	0.80	0.40	0.20	0.20	0.60	0.60	0.80	**Ava-I**	**0.096**
R-II	**0.34**	**0.35**	**0.40**	**0.25**	**0.27**	**0.28**	**0.31**	**0.47***	**0.43***	**0.28**	**0.35**	**0.45***	**0.30**	**0.29**	**0.40**	**0.46***	**0.47***	**0.32**	**0.40**	**0.40**	**0.60***	**0.80***		**0.39**

Average distances for both R-I & R-II = 0.486 or 48%

Table 2. Genetic distances for 23 pea lines calculated through UPGMA amalgamation rules using percent dis-agreement.

Lines	Genetic index (percent disagreement)			
	R-I	R-II	R-I & R-II	
			UPGMA value	Percentage
PL-1	0.05	0.34	0.39	39%
PL-2	0.06	0.35	0.41	41%
PL-3	0.06	0.4	0.10	10%
PL-4	0.06	0.25	0.31	31%
PL-5	0.07	0.27	0.34	34%
PL-6	0.07	0.28	0.35	35%
PL-7	0.08	0.31	0.39	39%
PL-8	0.08	0.47	0.55	55%
PL-9	0.48	0.43	0.91	91%
PL-10	0.05	0.28	0.33	33%
PL-11	0.05	0.35	0.40	40%
PL-12	0.24	0.45	0.49	49%
PL-13	0.04	0.3	0.07	07%
PL-14	0.04	0.29	0.33	33%
PL-15	0.05	0.4	0.09	09%
PL-16	0.06	0.46	0.52	52%
PL-17	0.07	0.47	0.54	54%
PL-18	0.08	0.32	0.40	40%
PL-19	0.1	0.4	0.5	50%
PL-20	0.13	0.4	0.17	17%
PL-21 (Pollen/Male)	0.2	0.6	0.8	80%
PL-22 Cultivar	0	0.8	0.8	80%
PL-23 (Female)	0.05	0.4	0.45	45%
Average	0.094	0.39	0.48	48%

R-I zone 1 starting from band 1- 5; Zone-II starting from band 6-14; R-I and R-II the average genetic distances calculated from band-1 to 14

Genetic association: The genetic association based on R-I of the parental (PL21 and PL-23), newly developed homo-lines (PL-01 to PL-20) and cultivars (PL-22) were tested via Two-ways Cluster plotting. In the tree-B, out of 23 lines the 19 (82.60%) were genetic linked in C-2, while 04/17.39 germplasm were grouped in C-1 namely PL-21, PL-20, PL-9 and PL-12 (Fig. 2).

The banding profile of R-II dissociate 23 lines into 4 clusters ©, the C-1 grouped 7/30.43% pea lines, which includes PL-22, PL-09, PL-12, PL-08, PL-15, PL-11 and PL-07. Similarly, C-03 were clustered 10/43.74% lines i.e. PL-17, 23, 21, 20, 13, 10, 14, 06, 05 and 04. The C-2 importantly grouped 2/8.69% germplasm i.e., PL-16 and PL-03. Furthermore, C-4 comprised of only 04/17.39% lines (PL-19, 18, 02 and 01) shown in Fig. 3.

Discussion

Protein electrophoresis using SDS-PAGE is one of the most reproducible, valuable techniques for studying estimation of genetic diversity at seed storage protein profile. This technique is particular reliable and the data scored during the process is largely independent of environmental fluctuations (Nisar *et al*., 2007). In Legumes several attempts have been done previously on electrophoresis patterns of the total seed protein through SDS-PAGE (Hajduch *et al*., 2005). In this study it was found that plant breeding through SSD method enhance 49% genetic diversity in pea, if all the population is well characterized and maintained from F-1 till ≥ F-10 population (Forster *et al*., 2014)

Higher the genetic diversity greater is a chance of improvement (Daniele *et al*., 2015). The same approach was applied in this experiment, it was deduced that 14 bands showed significant level of polymorphism with except of B-4 and B-5. Taxonomically, B-4 and B-5 were species specific bands in this experimental germplasm (Nisar *et al*., 2009; Tanuja & Singh, 2016). Genetic similarity (bands having low level of GD) among major bands indicates that the genes are highly conserved in peas. The highest level of genetic diversity was recoded in B-6, B-12, B-13 and B-14. The observed percentage of polymorphic bands was ranged in 45-91%, which is comparable to the finding of Ghafoor & Arshad, 2008. The polymorphism in R-II (49% GD) support the objectives of this work, which showed that mono-hybrid cross enhance the level of genetic diversity in edible pea. The variation in the protein bands reflects the genetic diversity caused by genetic change/structure, which expressed in seed storage protein. Generally, two methods namely; principal coordinate analysis and clustering are used for analyzing the genetic distance among various genotypes. Coordination is used to generate dimensional scatter plot of the genotypes, such that the distances among the genotypes in the plot reflect the genetic distances among them with a minimum of distortion. Another approach is to produce a dendrogram that discriminated various genotypes into different groups, which are genetically different from each other (Govindaraj *et al*., 2015). The same statistical analysis was carried out to find the genetic association and genetic distance among 23 pea lines. In zone-wise cluster plating, R-I the total germplasm were plotted in two clusters, which clearly showing that in this zone (B-1 to B5) the level of genetic linkage is higher with lower genetic disagreement. While, in R-II the germplasm were split in four clusters. Majorly, 43.74% lines were grouped in C-03 and C-02 only grouped 8.69% germplasm. While the remaining 30.43% and 17.39% were scattered in C-1 and C-4 respectively. Variation in band intensity and multiple clustering of germplasm validate that plant breeding enhances the level of genetic diversity based on seed storage protein profile in pea germplasm using SDS-PAGE. Similar trends were observed in the protein-banding pattern of other leguminous crops like cowpea, filed pea and also edible pea (Tanuja & Singh, 2016).

Acknowledgment

Authors acknowledge higher education commission (HEC) of Pakistan for provision of funds to carry out this research work under approved projects no: 5060, titled "Morphological and molecular cataloging of bean for the estimation of genetic diversity and selection of high yielding candidate lines".

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(Received for publication 17 November 2015)